EFFECT OF SUBSTRATE CONCENTRATION IN PRODUCING HIGHER BUTANOL COMPARED TO ETHANOL BY USING *CLOSTRIDIUM ACETOBUTYLICUM*

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ABSTRACT

In this present experiment, the main objective is to study the effect of substrate concentration in producing higher butanol compared to ethanol by using *Clostridium acetobutylicum.* Palm oil mill effluent (POME) was used as the main substrate in this fermentation because it imposes negative effect towards the environment which in vast amount besides the availability as a low cost substrate and reinforced clostridium medium (RCM) was used as a control substrate. Study was also done to investigate the growth profile of *C. acetobutylicum,* type of sugars in POME, and glucose consumption of *C. acetobutylicum* during fermentation. The HPLC analysis result for sugar component showed that the reducing sugars; fructose, glucose, galactose, sucrose and lactose exist in POME and are utilized as substrate for solvents fermentation by *C. acetobutylicum.* Main study on the effect of four substrate concentrations 70%, 80%, 90% and 100% in POME and RCM were tested using Schott bottle as fermentor in anaerobic chamber to maintain the anaerobic condition for *C. acetobutylicum* growth condition for 72 hours at temperature of 35^{o}C, pH 5.8 and speed of 200 rpm. The results showed that butanol and ethanol were produced at the end of the fermentation hence proving POME is a viable substrate for the fermentation. After 20 hours fermentation it was observed that at 90% substrate concentration butanol produced was higher compared to ethanol. However, in overall the result showed higher ethanol production compared to butanol production for all the four different substrate concentration throughout the experiment. The core factor contributing in this result is the substrate inhibition by butanol besides the phenolic component in POME which also acts as inhibitor, strain degeneration, and also extraction of butanol from fermentation broth. In conclusion, many efforts need to be taken to ensure higher butanol can be produced especially in decreasing the inhibition factor towards butanol.

ABSTRAK

Objektif dalam menjalankan kajian terbaru ini adalah mengkaji kesan kepekatan substrat dalam menghasilkan butanol yang lebih tinggi berbanding ethanol dengan menggunakan *Clostridium acetobutylicum.* Sisa buangan dari kilang memproses kelapa sawit (POME) digunakan sebagai substrat utama kerana sisa dalam jumlah sangat banyak ini mengakibatkan kesan negatif ke atas persekitaran selain kebolehsediaannya sebagai substrat berkos rendah dan 'Reinforced Clostridium Media' (RCM) digunakan sebagai substrat kontrol. Kajian juga dilakukan untuk mengkaji kadar profil pertumbuhan *C. acetobutylicum*, jenis gula dalam POME dan penggunaan glukosa oleh *C. acetobutylicum* semasa fermentasi. Keputusan analisa HPLC menunjukkan bahawa gula menurun; fruktosa, slukosa, galaktosa, sucrosa dan laktosa hadir dalam POME dan digunakan sebagai substrat untuk fermentasi. Kajian utama ke atas kesan empat kepekatan substrat yang berbeza 70%, 80%, 90% dan 100% dalam POME dan RCM dikaji menggunakan botol Schott dalam ruang anaerobik untuk mengekalkan keadaan anaerobik bagi pertumbuhan *C. acetobutylicum* selama 72 jam pada suhu 35^{o}C, pH 5.8 dan kelajuan 200rpm. Butanol dan ethanol terhasil dari fermentasi membuktikan POME adalah substrat yang sesuai untuk fermentasi ini. Selepas 20 jam fermentasi dapat diperhatikan bahawa pada kepekatan substrat 90%, butanol yang terhasil adalah lebih tingi berbanding ethanol. Walaubagaimanpun, secara keseluruhannya, ethanol dihasilkan lebih tinggi berbanding butanol di sepanjang eksperimen ini. Sebab utama yang menyumbang kepada keadaan ini adalah perencatan oleh butanol itu sendiri selain daripada komponen fenol dalam POME yang bertindak sebagai perencat. Selain itu, degenerasi strain dan juga penyaringan butanol dari pati fermentasi juga dilihat sebagai penyebab kepada keadaan di atas. Secara kesimpulannya, pelbagai usaha perlu dilaksanakan untuk memastikan penghasilan butanol yang lebih tinggi.

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LIST OF SYMBOLS / ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
BOD	-	Biological Oxygen Demand
C.acetobutylicum	-	*Clostridium acetobutylicum*
DNS reagent	-	Dinitrosalicylic Colorimetric Method
EFB	-	Empty Fruit Bunch
GC-FID	-	Gas Chromatography equipped with Flame Ionization Detector
HPLC	-	High Performance Liquid Chromatography
ME	-	Metabolic Engineering
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
OD	-	Optical Density
POME	-	Palm Oil Mill Effluent
RCM	-	Reinforced Clostridium Medium
RVP	-	Reid Vapor Pressure
SI	-	Spark Ignition
SS	-	Separator Sludge
USA	-	United States of America
UV-VIS	-	Ultraviolet-Visible Spectroscopy

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Under strain of human demand, oil prices have been fluctuating and resulted in the research on production of liquid fuels, such as butanol and ethanol, by fermentation. Deliberate actions have been taken towards production of alcohol fuels from easily and extensively-produced renewable resources prior to the constant conflict in oil-supply region of the world, and also the cascading decline of the fossil fuels.

The acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is one of the oldest known industrial fementations (second to ethanol) and is one of the largest biotechnological processes ever known. However, since the 1950's industrial ABE fermentation has declined continuously, and almost all butanol is now produced via petrochemical routes (Ramey & Yang, 2004). Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. The market demand is expected to increase dramatically if butanol can be produced economically from low-cost biomass (Durre, 1998).

Butanol is an alcohol that can be used as a transport fuel. It is a higher member of the series of straight chain alcohols with each molecule of butanol ($C_4H_{10}O$) containing four carbon atoms rather than two as in ethanol (Brekke, 2007). Because it is longer hydrocarbon chain causes it to be fairly non-polar, it is more similar to gasoline than it is to ethanol. Butanol which is produced from biomass such as molasses, corn, corn fiber, and other agricultural byproducts or processing wastes which require proper disposal to avoid pollution problems for example the palm oil mill effluents (POME) is known as "biobutanol". Both biobutanol and petrobutanol (from fossil fuels) have the same chemical properties.

Petroleum-derived butanol is currently used in food and cosmetic industries as an extractant. Bio-butanol is preferred, because there are concerns of its carcinogenic aspects associated with the residual petroleum components (Ramey, 2004). Butanol has the propensity to solve hydrogen infrastructure problems associated with fuel cell use of the future. Dispersed through existing pipelines and filling stations and then butanol can be reformed onboard the fuel cell vehicle, butanol offers a safer fuel with more hydrogen than methanol (very dangerous) or ethanol.

Palm oil mill effluent (POME) is a kind of byproduct of palm oil, but huge amount of it has been discarded in the vicinity of the palm oil mill plant. POME is a negative byproduct that might cause huge pollution of environment if not treated well before disposing it to the environment. Though, due to its high biological oxygen demands (BOD), it could be a kind of sustainable resource. Development of effective fermentation method for POME that contains fermentative sugars and fatty acid will make it real sustainable resource (Ngan *et al.*, 2003).

Acetone-butanol-ethanol (ABE) fermenting clostridia can catabolize various sustainable bio-resources including bio-wastes, due to its wide substrate specificities to various sugar substrate including cellulosic and hemicellulosic materials (Hayasida & Ahn, 1990). Glucose and fatty acid in POME could be expected as possible substrates for ABE fermenting clostridia. Moreover, ABE-clostridia, as anaerobes, do not require any aeration process or other facilities needed for aerobic fermentation system.

1.2 Problem Statement

Over the past decades, there has been heavy reliance on the fuels to be used in the cars. This is because cars make up the largest portion of the road and the percentage is increasing day by day. Oil prices have been fluctuating and resulted in the research on production of liquid fuels, such as butanol and ethanol, by fermentation. Deliberate actions have been taken towards production of alcohol fuels from easily and extensively-produced renewable resources prior to the constant cascading decline of the fossil fuels.

The need to produce fuel from raw material which is significantly less cost compared to petrochemical raw materials is very important. Therefore, these biomass based production will maintain or significantly increase in the demand in the market as the raw material used will be cheap and easy to be gained. Hence here the operating cost is directly reduced as POME is used because of the availability as a low cost raw material.

Besides that, industrialization is important to spur economic growth in a country. Malaysia is the largest producer and exporter of palm oil in the world. As a result of industrialization, palm oil manufacturing companies releases a big amount of effluent to the environment. The raw POME has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil, thus, POME should be treated before discharge to avoid serious environmental pollution. Raw POME has Biological Oxygen Demand (BOD) values averaging around 25,000 mg/litre, making it about 100 times more polluting than domestic sewage

Not only that, the main problem addressed here is the costly treatment of the waste. Treatment done by the Kualiti Alam Company at the moment is very costly. Hence the treatment to produce butanol is the best solution in reducing the cost of treatment. Where the focus is on changing the negative money, the money spent to treat something to positive money.

1.3 Objectives of study

To study the effect of substrate concentration in producing higher butanol compared to ethanol by using *Clostridium acetobutylicum.*

1.4 Scope of Study

To accomplish these objectives, the scope of work has been identified;

i. To study the growth profile of *Clostridium acetobutylicum*
ii. To complete the composition analysis of selected batch fresh POME by using HPLC
iii. To study the effect of substrate concentration on the higher butanol production compared to ethanol.
iv. To study glucose consumption in the fermentation broth.

CHAPTER 2

LITERATURE REVIEW

2.1 Fermentation

Fermentation is the conversion of a carbohydrate such as sugar into an acid or an alcohol. More specifically, fermentation can refer to the use of yeast to change sugar into alcohol or the use of bacteria to create lactic acid in certain foods. Fermentation occurs naturally in many different foods given the right conditions, and humans have intentionally made use of it for many thousands of years (McGuigan, 2009). The earliest uses of fermentation were most likely to create alcoholic beverages such as mead, wine, and beer.

Acetone-Butanol-Ethanol (ABE) fermentation is a process that uses bacterial fermentation to produce acetone, butanol and ethanol from starch. The process is anaerobic (it does not require oxygen), similar to how yeast ferments sugars to produce ethanol for wine, beer, or fuel. The process produces these solvents in a ratio of 3-6-1, or 3 parts acetone, 6 parts butanol and 1 part ethanol. It usually uses a strain of bacteria from the Clostridia Class (Clostridium Family) where *C. acetobutylicum* is the most well known strain. ABE fermentation for butanol production is gaining interest over the petrochemical route (Jones and Woods, 1986).

2.1.1 Anaerobic Fermentation

Anaerobic fermentation of carbohydrates by yeasts and bacteria leads to the production of a range of alcohols, acids and esters. Three alcohols, ethanol, isopropanol and butanol, are currently made industrially by fermentation, though, in most places, production from petroleum is cheaper than the biological conversion. The conversion of glucose to ethanol can be achieved at approaching the theoretical maximum efficiency of 51% (by mass) based on the biochemical route, retaining 93% of the energy content of the carbohydrate (Righelato, 1980).

2.2 Butanol over Ethanol

With the recent rise in oil prices to record levels alternative fuel sources are increasingly in demand. One option would be to switch to butanol produced by biological sources like bacteria using biomass as a fuel. This would mean using alternative fuels to power our cars, homes, appliances, computers and other oil dependant machines. The main sources of our current fuels (crude oil) are from fossils which are extracted from decayed bodies of ancient creatures.

The use of alcohol in spark ignition (SI) engines began in 1954 in countries like United States, Germany, and France. During World War I and II, gasoline shortages occurred in France and Germany, and alcohol was used in all types of vehicles, including military planes. Nowadays it is used with gasoline (a mixture) in the United States and has become a major fuel in Brazil (Nag, 2008). Any new fuel which is going to be introduced should be evaluated from the aspect of availability, renewability, safety, and cost adaptability to existing engines performance, economy and finally emission.

Butanol can generate energy when used in internal combustion engines similar to gasoline. However, for a variety of reasons it may actually be better than gas. Talking about its compatibility with existing vehicles, the air to fuel mixture ratio is 11.2 compared to gasoline which is 14.7. The energy content of Butanol is 105,000 Btu per gallon compared to gasoline's 114,000 Btu per gallon. This similarity between air to fuel mixture and energy content means conversion of existing vehicles would be very simple.

Butanol is a chemical with excellent fuel characteristics; butanol can solve many problems associated with the use of ethanol. Butanol has the following advantages over ethanol:

a) butanol has 25% more Btu per gallon
b) butanol is less evaporative/explosive with a Reid vapor pressure (RVP) 7.5 times
c) lower than ethanol
d) butanol is safer than ethanol because of its higher flash point and lower vapor
e) pressure
f) butanol has a higher octane rating
g) butanol is more miscible with gasoline and diesel fuel but less miscible with water.
h) butanol has energy density is only 10 to 20% lower than gasoline's.
i) It can be produced using existing ethanol production facilities with relatively minor modifications.
j) It is compatible with the current gasoline distribution infrastructure and would not require new or modified pipelines, blending facilities, storage tanks, or retail station pumps.

At the moment the main key challenge is the cost of the substrates used, the ability to use low cost substrates in producing cost effective butanol. Much work is being done to develop microbes that can be used with a variety of substrates. Palm **o**il **m**ill **e**ffluent is a potential exchange to the substrates used at the moment in the production of butanol as the main target of our world which is ‘Waste to Wealth’.

2.3 Palm Oil Mill Effluent

One of the components in the suspended solids of POME is separator sludge. Separator sludge acts as substrate to support production of solvents by *C. saccharoperbutylacetonicum* N1-4 without any need for mineral supplements. Besides that, enzymatic hydrolysis by cellulose prior to fermentation found to increase the yield of butanol by 75% (from 2.47g/l to 4.37g/l) (Mun *et al.*, 1995).

The current treatment technology of POME typically consists of biological aerobic and anaerobic digestion. Biologically treated effluent is disposed of via land application system, thus providing essential nutrients for growing plants (Wong *et al.*, 2002).

2.4 Solventogenic Clostridia

Clostridium is one of the largest bacterial genera with an enormous potential for biotechnical and medical applications. Despite growing scientific, medical, and industrial interest, information on basic methods, biochemical fundamentals, clinical practice, industrial applications, and novel developments remains scattered.

Solventogenic clostridia are strictly anaerobic, endospore forming bacteria that produce a large array of primary metabolites, like butanol, by anaerobically degrading simple and complex carbohydrates, including cellulose and hemicellulose (Papoutsakis, 2008). Solventogenic, butyric acid clostridia can produce a large array of metabolites, and metabolic engineering (ME) driven strain development could enhance these native capabilities and lead to production of chemicals including butyric and acetic acids, butanediol, propanol, and acetone (Jones & Woods, 1986).

2.4.1 *Clostridium acetobutylicum* in Butanol production.

In butanol production method, two types of microbes were used in two separate process steps. The first pass optimizes the production of hydrogen and butyric acid, while the second pass converts this acid into butanol. Each step utilizes a different Clostridium strain (Ramey, 2005).

Study by Monot *et al.* (1984) in the influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *C. acetobutylicum* showed that at lower pH, growth occurs in two consecutive phases and solvents are the main excreted metabolites. At the higher pH, there is a single growth phase with only acid formation. The influence of the pH can be correlated with a critical role of the concentration of undissociated butyric acid in the medium. Reducing the intracellular acid dissociation by lowering the intracellular pH also favours the production of acetone and butanol

POME fermentation using *C. acetobutylicum* NCIMB 13357 in an oscillatory flow bioreactor showed that POME is a viable media for ABE fermentation. Oscillatory flow bioreactor has an excellent potential as an alternative fermentation device. Fermentation was carried out for 72 hours at 35^{o}C using POME and reinforced clostridia medium as a growth medium in batch culture (Takriff *et al.*, 2009)

CHAPTER 3

METHODOLOGY

3.1 Introduction

Fermentation is is the main process to convert Palm Oil Mill Effluent (POME) to Butanol using Solventogenic Clostridia. There are few phases in this experiment before a product can be produced;

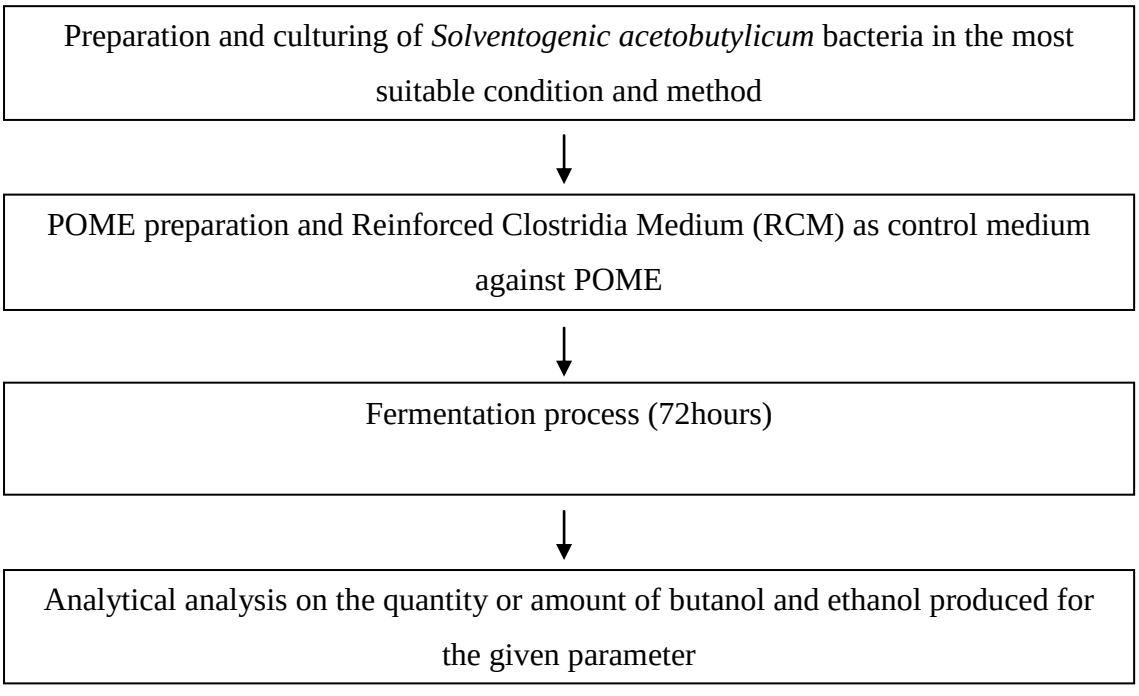

Figure 3.1: Flow Chart of experimental procedures

3.2 Material

3.2.1 Bacterial Strain

The bacterial strain, *C.acetobutylicum* NCIMB 13357 was obtained from University Kebangsaan Malaysia and used throughout the experiment. *C.acetobutylicum* is an anaerobic, saccharolytic and proteolytic bacterium that has been isolated from a number of environments.

3.2.2 Substrate

The fresh sample of palm oil mill effluent (POME) was obtained from Felda Palm Industries Sdn. Bhd., Lepar Hilir, Gambang, Kuantan. This sample consist of separator sludge which is the medium used for the experiment throughout. Separator **s**ludge contains fermentative sugars and fatty acids making it as a real sustainable resource.

3.2.3 Media

3.2.3.1 Reinforced Clostridium Medium

Reinforced Clostridium Medium that contained 5.0 g pancreatic digest of casein, 5.0 g proteose peptone, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g

dextrose, 5.0 g NaCl, 1.0 g soluble starch, 0.5 g cysteine hydrochloride, 3.0 g sodium acetate and 0.5 g agar per liter was used as the germination and inoculums media.

3.2.3.2 Reinforced Clostridium Agar

Reinforced Clostridium Agar that contained 10.0g casein enzymatic hylosate, 10.0g beef extract, 3.0g yeast extract, 5.0g dextrose, 5.0g NaCl, 1.0g soluble starch, 3.0g sodium acetate, 0.5g L-Cysteine hydrochloride and 13.5g agar per liter was also used as the agar medium for the growth of the bacteria culture.

3.3 Equipments

3.3.1 Anaerobic Chamber

The laboratory scale anaerobic chamber (Sheldon Manufacturing Inc., USA) was used for the anaerobic fermentation of *C.acetobutylicum* in POME.

3.3.2 Gas Chromatography – flame Ionization Detector

Gas Chromatography Agilent 6890 equipped with flame ionization (GC-FID) detector (Agilent Technology, USA) was used to detect the presence of butanol in

the fermentation product. The FID works by directing the gas phase output from the column into a hydrogen flame. A voltage of 100-200V is applied between the flame and an electrode located away from the flame. The increased current due to electrons emitted by burning carbon particles is then measured. The Specification of GC-FID that is used for product analysis is shown in Table 3.1.

Table 3.1: Specification of GC-FID for fermentation analysis

Specification of GC-FID	
Column	Hp-Innowax
Oven Temperature	$50^{o}C – 180^{o}C$
Carrier Gas	Hydrogen

3.3.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography Agilent 1200 (HPLC) (Agilent Technology, USA) was used for the analytical measurement of the sugar composition of selected batch fresh POME before the fermentation occurs. The Specification of HPLC that is used for product analysis is shown in Table 3.2

Table 3.2: Specification of HPLC for sugar analysis

Specification of HPLC	
Column	Supelcosil LC-NH_3
Injection Range	1ml/minute
Retention time	15minutes
Mobile Phase	75% Acetonitrile 25% Water
Standard Preparation	20 g/l ,40 g/l, 60 g/l, 80 g/l & 100 g/l for each sample of the standard

3.3.3 Ultraviolet-visible Spectrophotometer (UV-VIS)

Ultraviolet-visible Spectroscopy (UV-Vis) (HITACHI, Japan) refers to absorption spectroscopy in the UV-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum,molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

UV-VIS was used for the determination of glucose before, during each 20 hours of fermentation and after fermentation reading. The wavelength used for the glucose reading was 540 nm.

3.4 Experimental Procedure

3.4.1 Bacteria Culturing

3.4.1.1 Preparation of Agar Medium

Reinforce clostridia agar was prepared by dissolving 52.5 g of the powder in 1 liter of distilled water. After that, it was brought to the boil to dissolve the agar completely. Later agar medium was sterilized by autoclaving at 121^{o}C for 20 minutes. After autoclaving the medium, the medium was cooled at room temperature. Finally a tube of melted agar was poured into a sterile Petri dish and kept until the agar hardens.